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<b>(21) International Application Number:</b> PCT/US85/00054 <b>(22) International Filing Date:</b> 11 January 1985 (11.01.85) <b>(31) Priority Application Number:</b> 570,040 <b>(32) Priority Date:</b> 11 January 1984 (11.01.84) <b>(33) Priority Country:</b> US <b>(71) Applicant:</b> NEW YORK UNIVERSITY [US/US]; 70 Washington Square South, New York, NY 10012 (US). <b>(72) Inventor:</b> LEE-HUANG, Sylvia ; 345 East 69th Street, New York, NY 10021 (US). <b>(74) Agents:</b> GOGORIS, Adda, C. et al.; Darby & Darby, 405 Lexington Avenue, New York, NY 10174 (US).	<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> HUMAN ERYTHROPOIETIN cDNA CLONES  <b>(57) Abstract</b>  <p>A peptide immunochemically reactive with a monoclonal antibody to human erythropoietin, to a DNA fragment with a sequence coding for such peptide, to a recombinant DNA molecule having said DNA fragment inserted therein and to a transformed organism comprising an expression vector having said DNA molecule inserted therein, said organism being capable of producing said peptide, either directly or as a fusion protein. The present invention is also directed to methods for producing each of the above peptide, DNA fragment, DNA molecule and transformed organism.</p>		

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HUMAN ERYTHROPOIETIN cDNA CLONES

The United States Government has rights to this invention by virtue of grants No. RO1-HL21683 and No. RO1-HL30862 by the National Institute of Health, Bethesda, Maryland.

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Field of the Invention

The present invention relates to cDNA clones of human erythropoietin (Ep), to methods of identification and preparation of such clones, and to their expression products. More particularly, the present invention relates to:

20 (a) cDNA clones of human Ep identified from a cDNA-library constructed from human kidney mRNA, (b) synthesis of human Ep cDNA and insertion into pBR322 plasmids, (c) generation of E.coli expressing said cDNA, and (d) the products of expression of said cDNA.

25

The entire disclosure of my copending United States patent application Serial No. 570,075, filed on January 11, 1984 and entitled "REVERSE IMMUNOAFFINITY CHROMATOGRAPHY PURIFICATION METHOD" is hereby incorporated by reference as if fully set forth herein (said application being hereinafter referred to as the "Ep Purification Patent Application").

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The entire disclosure of my copending United States patent application Serial No. 570,039, filed on January 11, 1984 and entitled "MONOCLONAL ANTIBODY TO HUMAN URINARY ERYTHROPOIETIN AND HYBRIDOMA SECRETING SAID ANTIBODY" is hereby incorporated by reference as if fully set forth herein (said application

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1 being hereinafter referred to as the "Anti-Ep Patent Application"). Copies of extensive excerpts from both applications are attached as Appendix A and B, respectively.

#### Background of the Invention

5 A reliable and plentiful supply of human Ep has long been in demand to assist in a better understanding of the molecular mechanism of erythropoiesis and thus contribute to (a) the development of methods for the diagnosis and treatment of anemias and (b) the understanding of the  
10 differentiation and development of mammalian cells. Unavailability of sufficient human Ep has been due to scarcity of raw material, difficulties in its purification and lack of precise knowledge on the biogenesis of Ep.

The progress made by the present inventor in  
15 native human Ep purification (described in the Ep Purification Patent Application) by direct and reverse immunoaffinity chromatography, and in preparation of monoclonal Anti-Ep (described in the Anti-Ep Patent Application) has made it possible to attempt cloning of human Ep genes  
20 which, upon expression, can produce Ep protein.

#### Objects of the Invention

Accordingly, it is an object of this invention to provide an alternative source of human Ep protein.

Another object of this invention is to identify  
25 human Ep mRNA.

Yet another object of this invention is to synthesize and characterize human Ep cDNA.

Still another object of this invention is to produce a recombinant organism expressing human Ep cDNA.

30 A further object of this invention is to purify the product of said Ep cDNA expression.

A still further object of this invention is to characterize the sequence of human Ep cDNA and to identify the composition of human Ep.

35 These and other objects of this invention will be apparent to those skilled in the art in light of the present description, accompanying claims and appended drawings.

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### Summary of the Invention

One aspect of the present invention relates to a peptide immunochemically reactive with a monoclonal antibody to human erythropoietin.

Another aspect of the present invention relates to a DNA fragment coding for said peptide.

Yet another aspect of the present invention relates to a DNA molecule having inserted therein a DNA fragment comprising a DNA sequence coding for said peptide.

Still another aspect of this invention relates to a transformed living organism containing said DNA molecule said organism being capable of expressing said peptide.

Finally, this invention relates to methods for producing the above peptide, DNA fragment, DNA molecule and organism.

### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a fluorograph of electrophoresis of in vitro translation products of human kidney poly(A)<sup>+</sup> mRNA and immunoprecipitation of the translation products by monoclonal Anti-Ep.

Figure 2A depicts a silver stain of total proteins of human kidney extract, crude and pure Ep resolved by SDS-PAGE.

Figure 2B depicts an autoradiograph of an immunoblot from identical samples showing immunological detection of Ep-specific proteins.

Figure 3 is an autoradiograph of electrophoresis of the in vitro translation products of agarose gel size-fractionated mRNA.

Figure 4A is an autoradiograph of a nitrocellulose filter bearing positive colonies, as detected by colony hybridization.

Figure 4B is an autoradiograph of the same type of filter bearing a positive clone, pEp2, expressed in E.coli as a beta-lactamase fusion protein, detected by in situ radioimmunoassay using monoclonal Anti-Ep.

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1 Figure 5 is a photograph of agarose gel electrophoresis of cDNA inserts for positive Ep clones, visualized by ethidium bromide staining and ultraviolet transillumination.

5 Figure 6A is a fluorograph of electrophoresis of in vitro translation products from mRNA selected by hybridization with positive Ep clones.

Figure 6B is an Anti-Ep immunoblot of electrophoresis of in vitro translation products of mRNA selected by hybridization with positive Ep clones.

10 Figure 7 is an autoradiograph of competitive immunoprecipitation of hybrid selected translation products of M.W. 29,000 and 15,000 in the absence or presence of unlabeled Ep.

#### Detailed Description of the Invention

15 In order to clone Ep cDNA, its functional mRNA is first isolated. Difficulties in isolating this mRNA stem from the fact that the specific cellular site of Ep synthesis has not been firmly established, although the kidney is known to play a key role. Additional difficulties arise from the scarcity of viable human kidney samples and from their low Ep levels. It was therefore necessary to search for sources with elevated Ep levels and greater tissue availability.

25 It has been well documented that erythrocytosis may be associated with various renal tumors. Tumor extracts from some renal carcinoma patients have shown an increased level of Ep and such tumors were often considered as Ep-producing tumors. However, renal carcinoma tumors are only rarely accompanied by significantly increased Ep levels. Thus, the ability of renal carcinoma tissue to produce Ep after explantation (and in culture) is by no means assured. Accordingly, the present inventor engaged in an extensive search for human renal tumors showing increased Ep levels, in an effort to identify and secure a continuous source of functional Ep mRNA.

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1           In the work leading to the present invention, a  
total of thirty-six (36) renal cell carcinomas were exam-  
ined. Two of those demonstrated very high levels of Ep  
(0.9 and 3 units/g of tissue) by exhypoxic polycythemic  
5 mouse bioassay. It was observed that both normal tissue  
and cancerous tissue explants of carcinoma-affected kidneys  
sometimes displayed elevated Ep titers. By contrast,  
tissue explants of normal kidneys showed no detectable Ep  
activity by the same assay.

10           Prior to the present invention, there had been no  
specific procedure for the isolation of human kidney mRNA.  
Renal tissue is very rich in mRNA-inactivating ribonu-  
cleases (RNase) and is extremely difficult to homogenize.  
Further difficulties arise because of the limited availabil-  
15 ity of nephrectomy samples. Accordingly, conventional  
fractionation of tissue samples (into cytoplasmic, micro-  
somal and nuclear fractions) is impractical. Thus, total  
cellular RNA is preferably first isolated by extraction of  
disrupted kidney cells and messenger RNA is then selective-  
20 ly enriched, as follows:

Both normal and tumor portions from Ep-positive  
samples of renal carcinoma-affected tissues should be used  
for mRNA preparation. Throughout the procedure, care must  
be taken to minimize the degradative effect of (endogenous  
25 or exogenous) ribonuclease. Thus, preferably, RNase  
inhibitors are introduced in the extraction buffer.  
Reagents are sterilized and the glassware is baked at 250°C  
overnight prior to contact with RNA samples. Vanadyl-  
ribonucleoside (VRNS) complex or RNasin are preferred  
30 exogenous inhibitors of RNase, with VRNS being most pre-  
ferred. Preferably, the extraction buffer also includes a  
combination of chaotropic agents (such as ethylene glycol,  
propylene glycol or other common glycols) or dissociating  
agents (e.g., sarkosyl, guanidine hydrochloride and guan-  
idine isothiocyanate) and reducing agents (such as beta-  
35 mercaptoethanol). In general, the methods and reagents  
used significantly affect the yield and functionality of

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1 the mRNA obtained. Most preferably, the extraction buffer  
contains guanidine isothiocyanate, sarkosyl, beta-mercapto-  
ethanol, sodium citrate and vanadyl-ribonucleoside complex,  
5 which serve to disintegrate cellular structures, disso-  
ciate proteins and inactivate degradative enzymes and  
RNases.

The method of mRNA isolation employed in the  
present invention is fast, convenient and results in a good  
yield. The initial cell disruption is very important and  
10 it is preferably accomplished by dry-blending the tissue  
(as in an ordinary blender) in the presence of liquid  
nitrogen to ensure a sufficiently low temperature at which  
all enzymes will be inactive.

The powdered tissue is then treated with the  
15 extraction buffer. This is most conveniently and thorough-  
ly accomplished in the powdering blender. Dissolution of  
the cellular material and shearing of the DNA takes place  
by further blending, and passing the sample through a  
needle. Shearing is evidenced by a significant drop in  
20 viscosity of the mixture.

The general procedure of Ulrich et al: Rat  
Insulin Gene: Construction of Plasmid Containing the Coding  
Sequences, Science 196: 1313 (1977), was used with specific  
modifications as described in Example 2.

25 The concentration of total RNA is measured by  
absorbance at 260nm. The absorbance ratios 260/230 and  
260/280 are close to 280 are close to 2. This indicates no  
contamination of proteins and carbohydrates. A yield of  
about 0.5 to 1mg of RNA/gram of tissue is obtained.

30 Polyadenylated RNA (poly(A)<sup>+</sup> RNA) is then  
selected on an oligothymidylic acid-cellulose (oligo-dT  
cellulose) column as described by Aviv, H. et al, Purifica-  
tion of Biologically Active Globin Messenger RNA by Chroma-  
tography on Oligothymidylic Acid-Cellulose, Proc. Nat.  
35 Acad. Sci. (USA) 69:1408-1412 (1972). The poly(A)<sup>+</sup> RNA  
thus selected can be stored in ethanol at low temperature.  
Under these conditions, stability of the RNA is insured.

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1 In order to confirm that the poly(A)<sup>+</sup> RNA  
contains Ep message (functional Ep mRNA) some of the oligo  
dT-cellulose-selected mRNA is translated in vitro. Trans-  
lation may take place in a wheat germ cell-free system or,  
5 preferably, in a mRNA-dependent rabbit reticulocyte lysate  
system, as described by Pelham, H.R.B. et al, An Efficient  
mRNA-Dependent Translation System from Reticulocyte Lysates.  
Eur. J. Biochem. 67:247-256 (1976). The presence of mRNA  
coding for Ep protein in the poly(A)<sup>+</sup> kidney RNA is  
10 confirmed by immunoprecipitation of the translation  
products. Immunoprecipitation is carried out according to  
Kessler, S.W. Use of Protein-A Bearing Staphylococci for  
the Immune Precipitation and Isolation of Antigens from  
Cells: Meth. Enzymol. 73:442, 1981, with purified mono-  
15 clonal Anti-Ep IgG designated as 7A7. This specific  
procedure is described in Example 4. Two polypeptides are  
immunoprecipitated with monoclonal Anti-Ep. Their molecu-  
lar weights are 29,000 and 15,000 daltons, respectively  
(Fig. 1, lane 2). These polypeptides are not precipitated  
20 by preimmune mouse serum (Fig. 1, lane 3) nor are they  
detected in endogeneous translation and its immunopreci-  
pitated sample (Fig.1, lanes 6 and 7). The size of these  
polypeptides is smaller than expected for native Ep.  
However, in a rabbit reticulocyte lysate system, where the  
25 ribosomes are free in the cytosol and where endoplasmic  
reticulum membrane organization, and membrane bound enzymes  
are absent, post-translational modification such as glyco-  
sylation is not expected to occur. Thus, the 29,000 dalton  
polypeptide which is precipitated specifically by Anti-Ep,  
30 may represent the aglycosylated form of Ep. The 15,000  
dalton peptide may represent an immunologically related  
species. The presence of these polypeptides in tissue is  
also confirmed by immunoblotting of the tissue extract  
along with authentic Ep (Figure 2). The details of these  
35 experiments are described in Example 4.

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1           Some of the thus obtained mRNA is enriched in Ep  
mRNA by size fractionation on agarose gel in the presence  
of  $\text{CH}_3\text{HgOH}$  according to Bailey, J.M. et al, Methylmercury  
5           as a Reversible Denaturing Agent for Agarose Gel Electro-  
phoresis, Anal. Biochem. 70:75 (1976). The resolved RNA  
fractions are eluted and samples thereof are translated.  
The translation product is tested for Ep presence, prefer-  
ably by immunoprecipitation with monoclonal Anti-Ep ac-  
cording to the method of Kessler, supra.

10           The fraction of agarose-gel-fractionated RNA that  
was enriched in Ep mRNA (fraction 11 in Fig. 3) is reverse-  
transcribed into [ $^{32}\text{P}$ ]-labeled cDNA which is used as a  
hybridization probe in the identification of cDNA positive  
clones. Reverse transcription (using Avian myeloblastosis  
15           virus (AMV) reverse transcriptase) is preferable to T4  
kinase RNA phosphorylation treatment because it gives a  
higher specific radioactivity (of the order of  $10^7$   
cpm/ microgram) and it is less likely to label single-  
stranded contaminating ribosomal or tRNAs.

20           The remainder of poly(A)<sup>+</sup> kidney mRNA is used  
as the template for the synthesis of the 1st strand of cDNA  
by AMV reverse transcriptase in the presence of oligo-  
dT<sub>(12-18)</sub> as the primer. In view of: (a) the very rare  
opportunity presented in this case, i.e. the availability  
25           of a human kidney sample with significantly elevated Ep  
activity. and (b) the difficulties encountered in the  
preparation of intact polysomes from RNase-rich renal  
tissues, construction of a total human kidney cDNA library  
was undertaken. This approach not only entails minimum  
30           handling of the limited amounts of valuable RNA, but also  
allows other renal proteins of medical interest to be  
screened. Such a cDNA library would, of course, also  
require the screening of a large number of recombinants  
for Ep clones.

35           Conditions optimal for reverse transcription are  
highly dependent on the particular type of mRNA. The

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1 purity of the reverse transcriptase, its RNase contamination,  
the ratio of reverse transcriptase activity to mRNA  
quantity, the substrate concentrations, the pH and ionic  
5 factors which influence transcription efficiencies.

A ratio of 6 units of reverse transcriptase per  
microgram of mRNA template is preferred. Vanadyl ribo-  
nucleoside complex is preferably added to the transcription  
reaction mixture to inactivate contaminating RNase. The  
10 most preferred reaction conditions are set forth in Example  
5. The general reverse transcription procedure is conducted  
according to Retzel, E.F. et al., Enzymatic Synthesis  
of DNA by the Avian Retrovirus Reverse Transcriptase in  
vitro, Biochem. 19:513-518, 1980.

15 At the end of the reaction period, template RNA  
is denatured preferably with  $\text{CH}_3\text{HgOH}$  to avoid interference  
of template mRNA in the synthesis of ds-cDNA.

Complementary cDNA is preferably synthesized  
using the large (Klenow) fragment of E.coli DNA polymerase  
20 I (which does not have the 5'  $\rightarrow$  3' exonuclease activity)  
according to the method of Efstratiadis, A., et al.,  
Enzymatic In Vitro Synthesis of Globin Genes, Cell, 7:279,  
1976, wherein the reverse transcriptase loop from the  
previous step serves as the primer.

25 The hairpin loop covalently linking the first and  
second strand of cDNA is cleaved by nuclease  $S_1$ . The  
amount of  $S_1$  required is determined by titration in pilot  
experiments using alkaline agarose gel electrophoresis,  
which permits the sizes of the DNA molecules to be visual-  
30 ized. The Klenow product behaves as a molecule twice the  
size of cDNA, and the blunt ended double stranded cDNA (ds  
cDNA) behaves as two molecules the size of the original  
cDNA strand. The optimum amount of  $S_1$  nuclease was found  
to be 2.5 units per microgram of ds cDNA in this case.

35 In this work, 60 micrograms of poly(A)<sup>+</sup> RNA  
yielded 5 micrograms of blunt-ended ds cDNA.

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1           The ds cDNA is inserted in the Pst I restriction  
endonuclease site (located in the ampicillin resistance  
gene) of pBR322 by homopolymeric dC:dG tailing according to  
Villa-Komaroff et al., A Bacterial Clone Synthesizing  
5     Proinsulin, Proc. Nat. Acad. Sci. (USA) 72:3727, 1978.  
The oligo(dC)-tailed cDNA and oligo(dG)-tailed vector are  
annealed preferably at a ratio of 1:2.

pBR322 is preferred because of its versatility as  
a plasmid cloning vector. It is under relaxed control and  
10   contains both ampicillin and tetracycline resistance genes.  
The Pst I cleavage site is a single cleavage site within  
the ampicillin resistance gene. Cloning into the Pst I  
site inactivates this gene. Thus, the clones will be  
ampicillin-sensitive. In addition, they should also be  
15   tetracycline-resistant, if they have taken up the plasmid.

The recombinant plasmids are transformed into  
E. coli C600 made competent to take up DNA by  $\text{CaCl}_2$  and  
heat shock as described by Mandel, M. et al. Calcium  
Dependent Bacteriophage DNA Infection, J. Mol. Biol.  
20   53:159, 1970.

Transformants are selected for tetracycline  
resistance and ampicillin sensitivity. Since, as mentioned  
above, the Pst I site is in the ampicillin resistance gene,  
transformants will be ampicillin-sensitive ( $\text{Amp}^S$ ) but  
25   tetracycline-resistant ( $\text{Tet}^R$ ).

E. coli C600 is preferably used as the recipient  
because it is a good host for large scale growth and  
purification of plasmids and can be transformed with high  
efficiency by use of a plasmid vector annealed to cDNA by  
30   dC:dG homopolymeric tailing.

For maximum efficiency of transformation, the  
bacterial culture should be in the logarithmic phase of  
growth and the cell density about  $5 \times 10^7$  cell/ml at the  
time of treatment with calcium chloride. Maintaining the  
35   cells on ice for 12-24 hours prior to the transformation  
significantly increases transformation efficiency.

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1           The optimum transformation reaction ratios are  
1-10 ng of cDNA to 100 microliters of cell suspension.  
Larger amounts of suspension or cDNA result in lower  
transformation efficiencies. Use of top agar as a trans-  
5           formation plating medium is preferred.

          Although there are several procedures for identi-  
fication of recombinant plasmids that incorporate Ep DNA,  
the colonies are preferably initially screened by colony  
hybridization using [<sup>32</sup>P] labeled cDNA synthesized from  
10           enriched Ep mRNA as the probe, followed by in situ colony  
radioimmunoassay (RIA) using purified monoclonal Anti-Ep  
IgG.

          Colony hybridization is preferably conducted  
according to the general method of Grunstein, M. et al.  
15           A Method for the Isolation of Cloned DNAs that Contain a  
Specific Gene, Proc. Nat. Acad. Sci.(USA) 72:3961, 1975  
here adapted for a smaller scale (in order to save  
materials, which are in short supply, while maintaining  
sensitivity of the screening procedure) by use of very  
20           small amounts of [<sup>32</sup>P]-labeled cDNA as a probe with high  
specific radioactivity and small nitrocellulose filters,  
thus permitting a large number of colonies to be screened  
at a time. Duplicate colonies of transformants are grown  
on small filters, colonies are lysed and the DNA is de-  
25           naturated (by alkali treatment). Cell debris are treated  
with proteinase K and the DNA is fixed to the filter by  
baking. The DNA is hybridized to the probe, which binds  
only to its complementary DNA and permits positive colonies  
to be identified by autoradiography. This procedure is  
30           very efficient and results in elimination of about 95% of  
the colonies from further screening.

          Positive colonies are picked and grown on nitrocel-  
lulose filters for further screening by in situ colony RIA.  
This method requires the expression of antigenic determin-  
35           ants, i.e., the expression of the cDNA inserted in pBR322  
to produce a fused polypeptide containing the appropriate

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1 antigenic site for Anti-Ep recognition. Given the avail-  
ability of monoclonal Anti-Ep, RIA is a natural screening  
method choice for identification of positive clones. RIA  
5 is a particularly sensitive technique requiring only a  
small quantity of antigen containing an antigenic deter-  
minant. The general method of Helfman, D.M. et al:  
Identification of Clones that Encode Chicken Tropomyosin  
by Direct Immunological Screening of a cDNA Expression  
Library, Proc. Nat. Acad. Sci. (USA) 80:31 (1983) was  
10 employed.

Many operational variations of RIA are possible  
(direct or indirect, using  $^{125}\text{I}$ -labeled-Anti-Ep or  
 $^{125}\text{I}$ -labeled second antibody). The binding capacity and  
purity of the antibody significantly affect RIA sensitivity.  
15 Thus, use of crude ascitic fluid or only partially purified  
antibody should be avoided.

Radioiodination of antibodies is preferably  
carried out by the lactose peroxidase enzyme beads method  
as disclosed by Marchalonis, J.J., An Enzymic Method for  
20 the Trace Iodination of Immunoglobulins and Other Proteins,  
Biochem. J. 113:299, 1969.

In this work, three positive clones were identi-  
fied and designated pEp1, 2 and 3. All three reacted  
consistently with monoclonal Anti-Ep 7A7 (both  $^{125}\text{I}$ -  
25 labeled and unlabeled).

RIA-positive clones are grown in culture.  
Plasmid DNAs are isolated and the size of their cDNA  
inserts is determined by digestion with Pst I restriction  
endonuclease followed by electrophoresis in 6% polyacryl-  
30 amide. Phage  $\phi\text{X174}$  RF DNA Hae III digested fragments are  
used as size markers. The sizes of the inserts are ap-  
proximately 1400, 600 and 200 base pairs (bp) for pEp1, 2,  
and 3 respectively.

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1 To further confirm that the positive clones indeed contain Ep sequences, hybridization selection of Ep-specific mRNA is performed as disclosed by Parnes, et al.

5 Mouse beta-2 Microglobulin cDNA Clones: A Screening

Procedure for cDNA Clones Corresponding to Rare mRNAs, Proc. Nat. Acad. Sci. (USA) 78:2253, 1981, modified as follows: Plasmid DNA is suspended in H<sub>2</sub>O at 1mg/ml, heat denatured in 0.25 N NaOH, neutralized and spotted on nitrocellulose. The filters containing DNA are hybridized with poly(A)<sup>+</sup> RNA. It is advisable to use relatively large amounts of mRNA for clear results. (2.5 micrograms poly(A)<sup>+</sup> RNA/microgram of plasmid DNA).

Hybrid-selected mRNA is eluted from the filters, and translated in vitro as described above. <sup>35</sup>S-labeled translation products are analyzed by SDS-PAGE and fluorography. All three clones hybrid selected mRNA which directed the synthesis of polypeptides of molecular weight 15,000, 29,000, 66,000 and possibly 92,000 daltons. Final confirmation of the identity of the polypeptides was made by immunoblotting and competitive immunoprecipitation. SDS-PAGE of in vitro translation products from hybrid-selected mRNA are electrophoretically transferred onto a sheet of nitrocellulose paper (0.45 micron pore size) for immunoblotting with purified monoclonal Anti-Ep 7A7 IgG (obtained as described in the Anti-Ep patent application). This procedure, disclosed by Towbin, H. et al: Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheet: Procedure and Some Applications, Proc. Nat. Acad. Sci. (USA) 76:4350 (1979), is described in detail in Example 9. Competitive immunoprecipitation is carried out in the presence of excess unlabeled pure Ep as given in Example 10. Of the hybrid selected in vitro synthesized polypeptides, the 29,000

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1, dalton and 15,000 dalton polypeptides react with Anti-Ep on immunoblots and by immunoprecipitation and compete with authentic Ep for antibody binding.

5 The molecular weight of native human urinary Ep is 34,000 daltons. However, immunoblotting and immunoprecipitation of in vitro translation products of total kidney mRNA and hybrid selected mRNA all result in two polypeptides of M.W. 29,000 daltons and 15,000 daltons. Immunoprecipitation of tissue extracts from the Ep-rich renal carcinoma samples also resulted in identification of these two polypeptides, in addition to the 34,000 daltons polypeptide. The 34,000 daltons polypeptide is identical in size to the native glycosylated Ep. By contrast, the 29k polypeptide is smaller than expected for native glycosylated Ep. It is believed that in vitro translation in the message-dependent rabbit reticulocyte lysate system accounts for the smaller molecular weight, as discussed above. The hybrid selected 29,000 dalton peptide which was recognized specifically by monoclonal Anti-Ep is believed to be the aglycosylated form of Ep. The 15,000 dalton peptide, clearly immunologically related to, and sharing the antigenic determinants with, the 29,000 dalton and 34,000 dalton peptide, is believed to be an Ep-related protein.

25 Judging from the molecular weight of the native human urinary Ep, the cDNA insert of clone pEp1 is within the range of the coding size while those of clones pEp2 and pEp3 are too short to encode the complete sequence of Ep. These DNAs are useful in the preparation of full-length human Ep cDNA sequences for expression.

30 Alternatively, a cDNA fragment located at the 3' end of the mRNA, as determined by restriction mapping, will be used as a primer of mRNA reverse transcription. Since the 3' primer is a specific Ep cDNA fragment, the only cDNA synthesized by this reverse transcription reaction will be Ep cDNA. Optimization of reverse transcription is expected to yield entire Ep cDNA.

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1 Sequencing will be performed according to the  
techniques described in detail by Maxam, A.M. et al, A New  
Method for Sequencing DNA, Proc. Nat. Acad. Sci. (USA)  
74:560, 1977 and Sanger, F., et al., DNA Sequencing with  
5 Chain Terminating Inhibitors, Proc. Nat. Acad. Sci.(USA)  
74:5463 (1977).

The following examples serve to illustrate the  
present invention without limiting its scope:

Materials and Sources:

10 agarose, and vanadyl-XTP's complex were from  
Bethesda Research Laboratories (BRL), Bethesda, MD.  
creatine phosphate, spermidine, essential amino  
acids, Hepes, calf thymus DNA, lysozyme, tetracycline,  
ampicillin, dithiothreitol, ethylenediamine triacetate  
15 (EDTA), hemin, and sodium deoxycholate were from Sigma  
Chemical Co., St. Louis, Mo.

restriction endonucleases, pBR322 plasmid, E. coli  
DNA polymerase I, nuclease S<sub>1</sub>, and phage ØX174  
DNA-Hae III were from New England Biolabs, Beverly,  
20 Mass.

nitrocellulose filters were from Millipore,  
Bedford, Mass.

proteinase K, and calf liver tRNA were from  
Boehringer Mannheim Biochemical, Indianapolis, Indiana.

25 avian myeloblastosis virus (AMV) reverse transcrip-  
tase was from Life Sciences, Inc., St. Petersburg,  
Florida.

Sephadex G100, and protein A were from Pharmacia,  
Piscataway, N.J.

30 1,4-piperazine-diethanesulfonic acid (PIPES),  
deionized formamide (dF), guanidine isothiocyanate,  
cesium chloride, and Sarkosyl were from Fluka Chemical  
Corp., Hauppauge, N.Y.

bovine serum albumin was from Schwartz-Mann  
35 Biochemical, Spring Valley, N.Y.

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1           oligo-dT primer, and oligodithymidylic cellulose  
were from Collaborative Research, Waltham, Mass.

          [<sup>35</sup>S]-Methionine, <sup>125</sup>I-goat anti-mouse IgG,  
5           and lactose peroxidase enzyme beads were from New  
England Nuclear Company, Boston, Mass.

          DNase was from Worthington, Freehold, N.J.

          nitrocellulose sheet were from Schleicher &  
Schuell, Keene, N.H.

          Triton X-100 (TM) detergent was from Eastman  
10          Kodak, Rochester, N.Y.

EXAMPLE 1:

Search for Human Renal Samples with High Ep Titer

          Over a period of two and a half years, an exten-  
15       sive search was conducted for surgical renal carcinoma  
samples with Ep activity. Tissue from nephrectomy was  
obtained as fresh as possible, separated into normal and  
tumor portions, and washed with sterile, ice-cold phosphate-  
buffered saline to remove blood and extraneous material.  
20       Small portions were set aside for Ep bioassay and for the  
establishment of continuous culture. The remainder was  
frozen quickly in liquid nitrogen and stored at -70°C for  
the isolation of Ep mRNA. Ep activity in the tissue  
extracts was assayed in vivo by the exhypoxic polycythemic  
25       mouse method. Tissue homogenates were prepared by powder-  
ing the sample in liquid nitrogen and extracting it with  
20 mM sodium phosphate, pH 7.8, (equal ratio, wt(g)/vol  
(ml)). The extract was then centrifuged at 30,000 x g for  
30 minutes to remove cell debris and the clear supernatant  
30       was used for Ep bioassay.

          Of 36 renal cell carcinoma extracts examined, two  
demonstrated high Ep activity (0.9 and 3.0 Ep units/ml),

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1 six showed moderate activity (0.1 to 0.7 Ep units/ml) and  
the rest had either marginal (<0.1 Ep units/ml) or unde-  
tectable Ep activity. Tissue extracts of normal kidney  
from autopsy samples generally showed no detectable Ep  
5 activity but occasionally showed an activity of less than  
0.05 units/ml. The increase in Ep activity in the renal  
carcinoma samples was found in both the normal and tumor  
portions of the kidney. The samples with high Ep bioactiv-  
ity were used for mRNA preparation.

10

EXAMPLE 2:Isolation and Purification Of Functional Human Kidney RNA

A nephrectomy sample from a patient suffering  
from renal carcinoma with erythrocytosis and elevated Ep  
15 activity (Ep positive) of 3.0 units/g of tissue, was  
selected from Example 1.

Both the normal and tumor portions of the renal  
tissues were (separately) used for mRNA preparation.

20 Total human kidney mRNA was isolated by the  
guanidine/ cesium chloride method of Ulrich et al, supra.  
RNA preparations were made from 10 g batches as needed.  
The frozen tissue was powdered in liquid nitrogen in a  
blender. 5 volumes of an extraction buffer were then  
added. The extraction buffer contained: 6M guanidine  
25 isothiocyanate/ 5mM sodium citrate pH 7.0/0.1 M beta-  
mercaptoethanol/0.5% Sarkosyl. The mixture was homogenized  
for an additional 3 min. DNA was sheared by passing the  
mixture through a 22 gauge needle. The solution was  
centrifuged at 10,000 rpm for 15 min and the supernatant  
30 was collected. Cesium chloride was added to 0.4 g/ml of  
solution and the resulting solution was layered onto a 5.7

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1 M CsCl cushion containing 0.1 M EDTA (pH 7.5) (3 ml cushion and 2 ml of tissue extract) in a Beckman SW 50.1 centrifuge tube. Centrifugation was at 25,000 rpm for 16 hr at 20°C. The RNA pellet was dissolved in 10mM Tris-HCl, pH 7.4/5 mM EDTA/1% SDS, extracted with phenol, chloroform/1-butanol (4:1, v/v) and precipitated with ethanol.

5 EXAMPLE 3:

Selection of Poly(A)<sup>+</sup> RNA

10 The total RNA of Example 2 was poly(A)<sup>+</sup> selected on an oligo-dT cellulose column (0.9 x 10cm; bed vol 6.4 ml). RNA was suspended in 10mM Tris-HCl, 0.5M NaCl buffer (pH 7.4) and loaded onto the column. Selectively adsorbed poly(A)<sup>+</sup> RNA was eluted with 10mM Tris-HCl, pH 7.4, containing 1mM EDTA and 0.1% SDS and precipitated with 15 2 vols of ethanol at -20°C for 24 hours. The pellet was washed with 70% ethanol, resuspended in water and stored at -20°C. Usually 0.5 to 1 mg of total RNA was obtained from each gram of renal tissue and about 20 µg of poly(A)<sup>+</sup> RNA was obtained per mg of total RNA.

20 EXAMPLE 4:

In Vitro Translation and Immunoprecipitation

In vitro translation was carried out in a message-dependent rabbit reticulocyte lysate system, using [<sup>35</sup>S]-methionine as a label (New England Nuclear, 1236 Ci/mmol). 25 The lysate was preabsorbed with purified IgG of monoclonal Anti-Ep (50 µg/ml) and cleared with fixed Staphylococcus aureus bearing protein A (250 µg/ml, IgGSorb, obtained from New England Enzyme Center, Boston, Mass.). The reaction mixture (25 µl) contained 1 to 5 µg/ml 30 poly(A)<sup>+</sup> RNA, [<sup>35</sup>S]-methionine (NEN) 55 µCi, Hepes (pH 7.6) 20mM, KCl 80mM, Mg(OAc)<sub>2</sub> 1.3 mM, and reticulocyte lysate 10 µl. Incubation was carried out at 37°C for 1 hr. The labeled translation products were immunoprecipitated with purified IgG of monoclonal antibody to human 35 Ep. Immunoprecipitation was carried out according to

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1 Kessler, supra, using 5 to 50  $\mu$ g of IgG. The reaction  
components were pre-absorbed with mouse serum and the  
incubation was carried out in 10 mM Tris-HCl, pH 8.2/0.15 M  
NaCl at 4°C for 24 hours. The immune complex was collected  
5 by 100 to 200  $\mu$ l of a 10% suspension of fixed Staphylo-  
coccus aureus bearing protein A by incubation at 4°C for 1  
hour. The precipitate was washed 6 times with 50 mM  
Tris-HCl, pH 7.4./0.15 M NaCl/1 mM EDTA/0.1% NaDodSO<sub>4</sub>/1%  
Na deoxycholate/1% Triton X-100 (Buffer W) and suspended in  
10 gel sample buffer for electrophoretic analysis. The  
translation products and their immunoprecipitated products  
were resolved by SDA-PAGE and analyzed by fluorography  
(Figure 1). Two immunospecific polypeptides were identi-  
fied, one migrating at about MW 29,000 and the other at  
15 about 15,000 daltons (lane 2). These polypeptides were not  
precipitated by mouse preimmune serum (lane 3) and they  
were not detected in endogeneous translation and in the  
immunoprecipitated sample of such translation (lanes 6  
and 7). As discussed above, the M.W. 29,000 peptide which  
20 was precipitated specifically by the monoclonal Anti-Ep,  
may represent the aglycosylated form of Ep. The M.W.  
15,000 peptide, clearly immunologically related, may  
represent a precursor or degradative fragment of the  
aglycosylated Ep. To verify the existence of these poly-  
25 peptides in the original tissue extract, immunoblotting was  
conducted. Crude and purified human urinary Ep were also  
included on the same blot so as to provide a direct  
comparison of their immunospecificity. A single poly-  
peptide of M.W. 34,000 was blotted from both the crude and  
30 purified Ep (Figure 2B, lanes 2 and 3), whereas three  
polypeptides were detected in the tissue extract, M.W.  
34,000, 29,000 and 16,000 daltons. The 34,000 dalton  
polypeptide is identical in size to the authentic gly-  
cosylated Ep, and the smaller polypeptides are likely  
35 candidates for the aglycosylated precursor or degraded  
forms of Ep. The presence of these immunospecific poly-

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1 peptides both in the tissue extract and in the in vitro  
translation products supports their identification as  
Ep-related forms and the presence of functional Ep mRNA.  
An identical set of unblotted gel samples was subjected to  
5 silver stain for analysis of their total proteins (Figure  
2A), with respect to their Ep-specific proteins (Figure  
2B). The immunospecificity of monoclonal 7A7 is self-  
evident. These results indicate that the monoclonal  
Anti-Ep recognizes native glycosylated Ep as well as  
10 aglycosylated Ep and its precursors and fragments.

The monoclonal antibody to human Ep used in this  
work was prepared as described in detail in the Anti-Ep  
patent application.

Purified IgG was used for all immunoprecipita-  
15 tion, immunoscreening, and immunoblotting reactions  
described in the present work.

EXAMPLE 5:

Synthesis and Cloning of Double Stranded cDNA

Poly(A)<sup>+</sup> kidney mRNA (20  $\mu$ g) from Example 3  
20 was used as template for cDNA enzymatic synthesis. Avian  
myeloblastosis virus (AMV) reverse transcriptase was used  
to synthesize the first strand of cDNA in the presence of  
oligo-dT<sub>12-18</sub> primer. The conditions described are for  
500  $\mu$ l reaction mixture with 20  $\mu$ g of template. The  
25 reaction mixture contains poly(A)<sup>+</sup> RNA template, 50 mM  
Tris, pH 8.3, 10 mM MgCl<sub>2</sub>, 100 mM KCl, 140  $\mu$ g/ml  
primer, 1mM CH<sub>3</sub>HgOH to denature RNA, 30 mM beta-mercapto-  
ethanol, 1 mM vanadyl sulfate ribonucleoside complex, 2 mM  
each of deoxyribonucleotides, and 120 units of reverse  
30 transcriptase. Incubation was at 42°C for 1 hour. Free  
nucleotides were removed by gel filtration over Sephadex  
G100. The RNA template was denatured by treatment with 12  
mM CH<sub>3</sub>HgOH. The second strand of cDNA was synthesized  
using the Klenow fragment of E. coli DNA Polymerase I. The  
35 reaction consisted of 100 mM Hepes, pH 6.8, 70mM KCl, 7mM  
MgCl<sub>2</sub>, 10mM DTT, 22.5mM beta-mercaptoethanol, 0.5mM of

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1 each deoxyribonucleotide, and 100 units of Klenow enzyme  
per 2 g of cDNA. The reaction was carried out at 15°C  
for 18 hours in a final volume of 1 ml. After phenol  
5 extraction, Sephadex G100 gel filtration and ethanol  
precipitation, the cDNA was treated with S<sub>1</sub> nuclease to  
cleave the hairpin loop at the 5' end of the second strand.  
The amount of S<sub>1</sub> required was titrated for each experi-  
ment in pilot reactions using alkaline agarose gel electro-  
10 phoresis. Optimum concentration was found to be 2.5 units  
of S<sub>1</sub> nuclease per ng of double stranded cDNA in this  
case. The reaction mixture also contained 30mM NaAc, pH  
4.6, 300mM NaCl, and 3mM ZnSO<sub>4</sub> and the incubation was  
37°C for 1 hour.

The cDNA was then treated with terminal trans-  
15 ferase and dCTP to add 10-15 residues to the 3' end. Pst  
I-digested pBR322 was similarly treated with terminal  
transferase and dGTP. The reaction mixture contains 140mM  
potassium cacodylate, pH 7.2, 0.5mM CoCl<sub>2</sub>, 240mM dCTP or  
dGTP, 1.5 mg/ml BSA and 6,000 u/ml terminal transferase.  
20 The reaction was carried out at 25°C for 15 min. The cDNA  
was inserted into the Pst I site of pBR322 by homopolymeric  
dC:dG tailing. The oligo (dC)-tailed cDNA and oligo(dG)-  
tailed vector were annealed at a molar ratio of 1:2 at  
42°C for 2 hours. The recombinant plasmids were trans-  
25 formed into E. coli strain C600 by CaCl<sub>2</sub> treatment and  
heat shock. Transformants were selected for tetracycline  
resistance (Tet<sup>R</sup>) and ampicillin sensitivity (Amp<sup>S</sup>).

One ml of overnight E. coli C600 culture was  
inoculated into 100ml of L broth in a 500 ml flask. The  
30 cells were grown at 37°C with vigorous shaking to a density  
of about 5x10<sup>7</sup> cells/ml. The culture was chilled on ice  
for 10 min. and then centrifuged at 4,000xg for 10 min at  
4°C. The supernatant was discarded and the cells were  
suspended in (1/5 of the original culture volume) ice-cold,  
35 sterile solution of 100mM CaCl<sub>2</sub> and 20mM sodium acetate,

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- 1 pH 6.5. The resulting suspension was kept on ice for 20  
min, then centrifuged again for 10 min. The supernatant  
was discarded and the cells were resuspended in (1/100 of  
5 of 0.1M  $\text{CaCl}_2$  and 20mM sodium acetate. The suspension  
was kept on ice for 20 hours.

- To 100  $\mu$ l of the cell suspension, 1-10 ng of  
vector were added in each of ten tubes. The mixture was  
left on ice for 10-30 minutes. 1 ml of LB broth was added  
10 to each tube and incubation was carried out at 37°C for one  
hour with shaking, to allow the bacteria to recover and to  
express antibiotic resistance.

- Transformants were selected for tetracycline  
resistance ( $\text{Tet}^R$ ) and ampicillin sensitivity ( $\text{Amp}^S$ ) on  
15 LB-Tet plates (10g bacto-tryptone, 5g yeast extract, 5g  
NaCl, 3.5 ml (1M) NaOH and 15g agar per liter containing 25  
 $\mu\text{g/ml}$  tetracycline), and on LB-Amp plates (containing 100  
 $\mu\text{g/ml}$  of ampicillin, instead of tetracycline). Colonies  
began to appear 12-16 hours after onset of 37°C incubation.  
20 The transformation frequency was  $5 \times 10^5$  transformants  
per microgram of cDNA on selection for tetracycline resis-  
tance. Approximately 95% of the transformants were both  
tetracycline-resistant and ampicillin-sensitive.

#### EXAMPLE 6:

- 25 Enrichment of Ep-mRNA and Synthesis of [ $^{32}\text{P}$ ] cDNA probe  
Methylmercury Hydroxide Agarose Gel Electrophoresis  
Poly(A) $^+$  RNA (50  $\mu\text{g}$ ) was fractionated by  
electrophoresis in a 1.5% agarose gel containing 12.5 mM  
CH<sub>3</sub>HgOH using low melt agarose (BRL, Bethesda, MD), at 40  
30 volts for 15 hours. Gel lanes containing size markers  
( $\phi$ X174 Hae III digest and ribosomal RNA's of HeLa cells)  
were soaked in 0.5 M ammonium acetate and stained with  
ethidium bromide. Gel lanes containing poly (A) $^+$  were  
soaked in 100 mM DTT to allow renaturation of RNA. The  
35 lanes were sliced into 30 fractions and the RNA was ex-  
tracted from these fractions by controlled microwave

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1 heating and phenol extraction, followed by ethanol precipi-  
tation. Portions of the fractionated RNA's were translated  
in vitro and immunoprecipitation of the translation pro-  
ducts was carried out as described in Example 4 to locate  
5 the fraction(s) enriched in Ep mRNA. The <sup>35</sup>S-labeled  
translation products and their immunoprecipitates were  
analyzed by SDS-PAGE. The majority of Ep mRNA was resolved  
in fraction number 11 (Figure 3, lane 11), as detected by  
immunoprecipitation of the translation products with  
10 Anti-Ep 7A7 (Figure 1, lanes (4) and (5)). Using ribosomal  
RNA and ØXDNA Hae III fragments as markers, fraction  
number 11 corresponds approximately to 1400 bp in size.  
This fraction was used to synthesize <sup>32</sup>P-labeled single-  
stranded cDNA according to Example 5. A specific radio-  
15 activity of 10<sup>7</sup> cpm/μg was obtained. The <sup>32</sup>P-labeled  
cDNA was used as a probe for the initial screening of  
recombinant plasmids.

EXAMPLE 7:

Initial Screening of the cDNA Library:

20 In Situ Colony Hybridization

Tet<sup>R</sup>, Amp<sup>S</sup> transformants from Example 5 were  
individually picked and grown on nitrocellulose filters  
(Millipore, 4.5 cm containing 100 gridded squares). 100  
colonies were inoculated onto each of 12 filters, each  
25 colony within a grid square. Each filter was placed on the  
surface of an LB-Tet plate and incubated at 37°C for 20  
hours. The transformants were screened by a modification  
of colony hybridization using <sup>32</sup>P-labeled cDNA synthe-  
sized from size-fractionated mRNA enriched in Ep message.

30 Filters bearing colonies were treated with 0.4  
N NaOH, neutralized, and treated with proteinase K. The  
DNA was fixed to the filter by baking at 80°C in vacuo for  
4 hours. Twelve filters were hybridized together in 3 ml of  
probe at 1 x 10<sup>6</sup> cpm/ml in 50% deionized formamide (DF)  
35 and 0.75M NaCl/75mM sodium citrate at 37°C for 24 hours.  
The filters were washed six times with 0.3M NaCl/30mM

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1 sodium citrate at room temperature (40 min./wash), blotted  
dry, and exposed to film.

5 A typical filter bearing the colony hybridization  
results is shown in Figure 4A, and two classes of positive  
colonies were detected. One, comprising 0.1 to 0.4% of the  
total colonies, hybridized very strongly to the probe and  
exhibited dense spots on autoradiography. The second  
10 class, which consisted of 5% of the total colonies, hybrid-  
ized to the probe in varying degrees but significantly less  
strongly than did the first class. The remainder of the  
colonies were negative. By this preliminary screening,  
about 95% of the transformants were eliminated from further  
screening.

EXAMPLE 8:

15 Immunological Screening of Colonies  
and Characterization of Plasmid DNA

Immunological screening was carried out by in  
situ colony radioimmunoassay (RIA) with monoclonal anti-  
bodies to human Ep using the general method of Helfman et  
20 al., supra. Bacterial colonies were grown on 4.5 cm  
nitrocellulose filters as described above, and lysed over  
CHCl<sub>3</sub> vapor for 30 minutes. Each filter was treated with  
10 ml of lysis buffer in a Petri dish at room temperature  
overnight with gentle shaking. The lysis buffer contains  
25 3% bovine serum albumin (BSA), 50 µg/ml lysozyme, 2 µg/ml  
DNase in 50mM Tris HCl pH 7.4./150 mM NaCl (Tris/saline).  
The filter was rinsed thoroughly with Tris/ saline and  
incubated at room temperature for one hour with 5 ml  
of purified IgG 7A7 (1mg/ml) in Tris/saline/3% BSA. The  
30 filters were washed six times with the same buffer at 45  
minutes per wash to remove nonspecifically adsorbed anti-  
body. Bound antibody was detected by a one hour incubation  
with <sup>125</sup>I-labeled affinity purified goat anti-mouse IgG

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1 (1 x 10<sup>6</sup> cpm/ml). The filters were washed extensively  
(six to eight washes) with buffer W, and analyzed by  
autoradiography. Positive clones were verified by direct  
colony RIA using <sup>125</sup>I-labeled monoclonal anti-Ep (2 x  
5 10<sup>6</sup> cpm/ml). Radioiodination was carried out by the  
lactose peroxidase method. In all RIA's, a filter spotted  
with various amounts of purified Ep was included as a  
control for the specificity of the immunological detection  
of the antigen. In such controls, <1 ng of purified Ep  
10 could be detected.

Positive recombinants from colony hybridization  
were picked, and grown on gridded nitrocellulose filters in  
a registered fashion for immunological screening by in situ  
colony RIA with 7A7. This procedure relies on expression  
15 of the cDNA inserted in the pBR322 beta-lactamase operon to  
produce a fused polypeptide containing the appropriate  
antigenic site for the Anti-Ep recognition. From 1.4 x  
10<sup>5</sup> transformants, three positive clones were identified  
that reacted consistently with 7A7. These clones were  
20 designated pEp1, 2, and 3. A representative filter showing  
the detection of such a positive clone (pEp2) is seen in  
Figure 4B. The immuno-specificity of the positive clones  
was further confirmed by direct colony RIA using <sup>125</sup>I-  
labeled 7A7. All three clones reacted positively and  
25 consistently with [<sup>125</sup>I]7A7. The plasmid DNA of these  
clones was isolated and its size determined, as described  
below.

Plasmid DNA was prepared by the alkaline lysis  
procedure of Birnboim, H.C. et al, A Rapid Alkaline Extrac-  
30 tion Procedure for Screening Recombinant Plasmid DNA, Nuc.  
Acid Res. 7:1513, 1979, and purified by centrifugation  
through 1M NaCl in a SW 50.1 rotor at 40,000 rpm for 6  
hours at 20°C. Digestion with restriction endonucleases  
was carried out under conditions recommended by the sup-  
plier. Gel electrophoresis was carried out in 6% poly-  
35 acrylamide gels or in 1% agarose gel. ØX174 RF DNA -

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1 Hae III digested fragments were used as markers. The size,  
of the inserts are 1,400, 600 and 200 base pairs for pEp1,  
2 and 3 respectively (Figure 5, lanes 2, 4 and 1 respec-  
tively).

5 EXAMPLE 9:

Hybridization Selection of mRNA and In Vitro Translation

Selection of mRNA was conducted according to the  
procedures modified from Parnes et al., supra. Plasmid DNA  
was suspended in H<sub>2</sub>O at 1 mg/ml, denatured by heating 2  
10 minutes at 96°C in 0.25 N NaOH, cooled quickly, neutralized  
with HCl, and spotted to saturation on nitrocellulose. The  
filters were baked for 2 hours at 90°C and then hybridized  
with human kidney poly(A)<sup>+</sup> RNA at a ratio of 2.5 µg  
poly(A)<sup>+</sup> RNA per µg of plasmid DNA. Hybridization was  
15 carried out at 50°C for 3 hours in a total volume of 100  
µl containing 65% (v/v) DE/20 mM 1,4-piperazine-diethanesul-  
fonic acid (PIPES), pH 6.4/0.2% SDS/ 0.4. M NaCl / 100  
µg per ml calf liver tRNA. The hybrid selected mRNA was  
eluted with 200 µl of H<sub>2</sub>O at 100°C for 90 seconds and  
20 then snap frozen in liquid nitrogen. The eluate was  
precipitated with ethanol using 10 to 20 µg of calf liver  
tRNA as a carrier. The hybrid selected mRNA was translated  
in vitro and the <sup>35</sup>S-labeled translation products were  
resolved by SDS-PAGE and analyzed by fluorography (Figure  
25 6). As seen in Figure 6A the RNAs selected by pEp1, 2 and  
3, all directed the synthesis of four <sup>35</sup>S-labeled polypep-  
tides (lanes 4, 5 and 6 respectively). The molecular  
weight of these polypeptides relative to the gel markers  
were approximately 92,000, 66,000, 29,000 and 15,000  
30 daltons. The 92,000 dalton band was also seen in the  
endogeneous translation (lane 0) and in the pBR322 selected  
sample (lane 1) but at a much lesser intensity. The  
66,000, 29,000, and 15,000 dalton polypeptides were not  
detected in lanes (0) and (1), indicating that they are  
35 Ep-specific.

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1 Final confirmation of the identity of these polypeptides was made by immunoblotting and competitive immunoprecipitation as described in Example 10.

EXAMPLE 10:

5 Detection of Antigenic Proteins Synthesized in vitro by Immunoblotting, and Competitive Immunoprecipitation

Electrophoretic transfer of proteins was carried out according to the general procedure of Towbin et al., supra. Nitrocellulose, 0.45  $\mu$ m pore size was used. The transfer was conducted in 25mM Tris HCl pH 8.4/192 mM glycine /20% methanol (v/v) at 0.35 amperes for 12 hours and then at 1 ampere for 3 hours using a Hoefer TE42 Transphor unit with a TE 50 power supply. The electrophoretic blot was rinsed with Tris/saline and incubated with 3% BSA/Tris/saline for 1 hour at 40°C to saturate the remaining protein binding sites. It was then incubated with 7A7 (1 mg/ml) in 3% BSA/Tris/saline for 1 hour at room temperature or overnight at 4°C. After washing with Tris/saline, the blot was incubated with  $^{125}$ I-labeled goat anti-mouse IgG ( $1.2 \times 10^6$  cpm/ml) for 1 hour at room temperature. For every 100 cm<sup>2</sup> blot, 10 ml of the antibody solution was used. The blot was thoroughly washed with buffer W six to ten times at 45 minutes per wash and exposed to film. The results are seen in Figure 6B. Two polypeptides, M.W. 29,000 and 15,000 were immuno-blotted by the Anti-Ep whereas the M.W. 92,000 and 66,000 polypeptides were not detected. This is true in the translation of total kidney poly(A)<sup>+</sup> RNA (lanes 2 and 3) as well as in the hybrid selected mRNA (lanes 4, 5 and 6). Whether this is due to inefficient transfer of high molecular weight proteins or a lack of antigenic recognition remains to be studied.

Competitive immunoprecipitation of  $^{35}$ S-labeled, hybrid selected translation products was carried out by the addition of purified unlabeled native Ep to the immune reaction mixture. Both the  $^{35}$ S-labeled M.W. 29,000 and

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1 15,000 polypeptides were immunoprecipitated by 7A7 in the  
absence of unlabeled Ep (Figure 7, lane 3). Precipitation  
of these polypeptides was inhibited by the addition of  
excess unlabeled Ep (lanes 4 and 5). Preimmune mouse serum  
5 did not precipitate these polypeptides (lane 1) nor was any  
precipitation detected in endogeneous translation (lane 2).  
About 50% inhibition resulted in the presence of 2  $\mu$ g of  
unlabeled purified Ep (lane 4) and over 90% of inhibition  
was observed upon the addition of 10  $\mu$ g of purified  
10 unlabeled Ep (lane 5). These results indicate that of the  
hybrid selected translation products, the 29,000 and  
15,000 dalton polypeptides are recognized by monoclonal  
Anti-Ep, 7A7 and that authentic Ep competes with them for  
antibody binding.

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APPENDIX AEp Purification

According to the present method, unconcentrated or concentrated urine from severely anemic patients can be used as the raw material. Starting samples, are preferably first centrifuged to eliminate insoluble material and purified preferably by hydrophobic interaction chromatography (HIC), as described by Lee-Huang, S.: A New Preparative Method for the Isolation of Human Erythropoietin With Hydrophobic Interaction Chromatography, Blood 56:620-624, 1980, in order to remove the bulk of urinary contaminants and permit more efficient and repeated use of the immuno-adsorbents. HIC involves processing of the raw material through a crosslinked neutral gel chromatographic column wherein the gel contains a hydrophobic group. Phenyl-Sepharose CL4B is particularly preferred because it provides a strong yet easily reversible binding with Ep. Octyl-Sepharose may also be used, but Ep elution therefrom is less complete. The specific activity of Ep obtained from this step depends on the potency of the starting material but generally ranges between about 115 and 250 units per mg of protein. The yield is usually about 80%. One unit of Ep is defined as the activity contained in 0.5 mg of the second International Reference Preparation of Human Urinary Erythropoietin (IRP) (obtained from the World Health Organization, International Laboratories of Biological Standards, Hampstead, London, England), or one-tenth of the contents of one ampule of this preparation.

The HIC-purified material can be used as the immunogen to raise antibodies to Ep (hereinafter designated as "Anti-Ep") and its common contaminating impurities (hereinafter designated as "Anti-I"). This can be conveniently performed in a single immunization using antibody-producing laboratory animals. The immunization is carried out in accordance with methods well known in the art and, in the case of Ep or other weak immunogens, it preferably includes several booster injections in addition to the

-30-

1 initial injection. Anti-Ep titers are determined by the in  
vivo exhypoxic polycythemic mouse bioassay described by  
Camiscoli, J.F. and Gordon, A.S.: Bioassay and Standardi-  
5 zation of Erythropoietin in Gordon, A.S. (Ed.) Regulation  
of Hematopoiesis, Meredith Corp., New York, 1970 pp 370-396.

Polycythemia is induced in mice by hypobaric hypoxia. In order to keep a high protein concentration and thus stabilize the Ep activity, Ep samples for assay are made up in a buffered albumin solution. Samples are in-  
10 jected into mice posthypoxia, intraperitoneally. Ep activity is measured by its stimulation of  $^{59}\text{Fe}$  incorporation in red blood cells.  $^{59}\text{Fe}$  incorporation is determined in a gamma counter. The results are compared to those obtained using the second IRP from WHO. Anti-Ep  
15 titers are determined by assaying for ability to neutralize Ep-stimulated  $^{59}\text{Fe}$  incorporation in red blood cells.

The immunized laboratory animals are then finally bled. Antisera from the bleedings after the last injection are isolated, assayed for anti-Ep titers, and purified by  
20 immunoaffinity chromatography to eliminate non-immunoglobulins. The rabbit antisera are processed through a Sepharose 4B column to which goat-(anti-rabbit) Igs have been covalently linked. The non-immunoglobulins are excluded from the column, while the specific Igs are eluted with, e.g.,  
25 3M sodium thiocyanate (NaSCN) or 0.2M acetic acid.

The thus obtained specific immunoglobulin preparation is treated to separate Anti-Ep from Anti-I. For this purpose, a highly purified Ep preparation is preferably used. However, the present invention does not require pure  
30 Ep for antibody preparation and/or separation. Partially purified Ep (or other partially purified antigen), prepared according to conventional methods, is adequate for carrying out the method of the present invention.

The antibody separation may be preferably accomplished by a new principle and procedure which employs  
35 reversible binding of antigen to a supporting matrix and

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1 thus permits subsequent recovery of valuable Ep (or other antigen) after it is used in the Anti-Ep (or other antibody) purification, without substantial loss of activity.

5 The antibody separation procedure utilizes the fact that Wheat germ Lectin-Sepharose 4B (WGLS) columns coated with purified Ep have differential affinity for their biospecific and immunospecific ligands. The procedure involves four steps:

10 1. Purified Ep is bound to WGLS to produce a WGLS-Ep complex. Ep binds tightly to WGLS due to interaction of its N-acetyl-glucosaminyl residues with the wheat germ lectin.

15 2. The affinity purified rabbit immunoglobulins are processed through a WGLS-Ep column: Anti-Ep binds to the WGLS-Ep complex, while Anti-I does not, but is excluded in the effluent and set aside for further use. Of course, since the original purified Ep, that was used to coat the WGLS column in Step 1, was not homogeneous, its impurities will also be carried over to the WGLS column of Step 1 and, consequently a small fraction of the Anti-I will bind to the WGLS-Ep column of Step 1. This was the shortcoming of conventional immunoaffinity techniques which the present invention has overcome, as will be described below.

20 Anti-Ep bound to the WGLS-Ep complex is eluted and preferably processed again through a regenerated WGLS-Ep column to insure complete resolution of Anti-Ep/Anti-I immunoglobulins. The Anti-I-containing eluents from the first and the second separation are pooled and Anti-I are recovered therefrom.

30 3. Since the affinity between the constituents of the immune complex (Ep-(Anti-Ep)) is lower than the affinity between Ep and the sugar-lectin complex (WGLS-Ep), Anti-Ep from WGLS-Ep-(Anti-Ep) can be selectively eluted using a weak acid or a dissociation reagent. The ability of WGLS to bind Ep both at low pH and under dissociating conditions makes WGLS a useful adsorbent for Anti-Ep

35

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1 purification and at the same time enables recovery of the  
valuable Ep (see step 4 below). The thus recovered Anti-  
Ep is separated from the eluent (e.g., by dialysis) lyophi-  
lized, and stored frozen for subsequent use.

5 4. Ep can be recovered from WGLS-Ep, once Anti-  
Ep has been eluted, by further elution, preferably with  
N-acetylglucosamine or N,N-diacetylchitobiose. This is not  
possible under conventional immunoaffinity procedures  
since, normally, the immunoabsorbent is irreversibly  
10 coupled to the supporting matrix and cannot be recovered.  
When the supply of the antigen (used as the immunoabsorbent)  
is limited, the recovery of such materials is a very  
valuable saving. Alternatively, the column of WGLS-Ep can  
be regenerated and can be reused.

15 The thus recovered Anti-Ep and Anti-I are sepa-  
rately covalently linked to CNBr-activated Sepharose 4B.  
The coupling procedure has been generally described by  
Axen, R. et al "Chemical Coupling of Peptides and Proteins  
to Polysaccharides by Means of Cyanogen Halides" Nature,  
20 214:1302-1304, 1967. The Sepharose-(Anti-Ep) and Sepharose-  
(Anti I) so prepared are used in column form for the direct  
immunoaffinity chromatography (DIAC) and reversed immunoaf-  
finity chromatography (RIAC) purification of Ep.

25 The Ep purified by HIC is further purified by  
DIAC on a Sepharose-(Anti-Ep) column. This purification  
results in exclusion of the majority of contaminants from  
the column, which are carried off in the effluent, while Ep  
is retained on the column. It is important to note, how-  
ever, that at this stage some antibodies to some minor  
30 impurities will be present in the Sepharose-(Anti-Ep)  
column because of the lack of homogeneous Ep in the immuno-  
affinity purification of the Anti-Ep. This is the intrinsic  
limitation of any conventional direct immunoaffinity  
technique.

35 Ep from the Sepharose-(Anti-Ep) column is eluted  
with an appropriate buffer. Choice of buffer is important

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1 in preserving Ep activity. For example, commonly used  
immune complex dissociating acidic buffers or chaotropic  
ions (such as glycine hydrochloride buffer or sodium thio-  
cyanate) inactivate Ep, while simple alkali gives incomplete  
5 desorption. The present inventor has found that inclusion  
of 10-20% of a polarity reducing agent (such as glycerol or  
another common 1,2-glycol) and a dissociation agent (such  
as guanidine hydrochloride or urea) in an alkaline eluant  
(such as NaOH) facilitates effective release of Ep from the  
10 immunoabsorbent while preserving Ep activity. Preferred  
are ethylene glycol and guanidine hydrochloride, which can  
be easily removed and which appear to have no detrimental  
effect on Ep activity.

The thus eluted Ep is dialyzed (preferably immedi-  
15 ately and thoroughly) against water and sodium phosphate  
buffer. Under these conditions, DIAC is very efficient,  
offering a high purification factor (usually about 169-fold  
over HIC) and a high yield (usually about 80% or higher).  
However, the main limitation of DIAC is the impurities in  
20 the original Ep preparation. The antibodies against these  
impurities are carried over in the purification system and  
immunoabsorb their antigens in the Sepharose-(Anti-Ep)  
column. As a consequence, the purity of the DIAC product  
cannot exceed that of the original Ep used in preparation  
25 of the WGLS column (Step 1) for antibody purification.

At this point further purification is accomplished  
with another Sepharose column coupled with Anti-I. While  
Anti-Ep contains only a minor fraction of antibodies to the  
impurities, Anti-I consists of the bulk of these antibodies.  
30 Thus, the Sepharose-(Anti-I) column will be able to provide  
sufficient antibody sites to bind substantially all the  
impurities contained in the DIAC-purified Ep.

Upon loading DIAC-purified Ep onto a Sepharose-  
(Anti-I) column, the trace impurities are retained in the  
35 column due to the formation of specific immune complexes  
with their corresponding antibodies, which are present in

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1 great excess on the column, whereas pure Ep is selectively  
excluded in the effluent. This step affords preparation of  
Ep which is purier than the original antigen. Such effi-  
5 ciency is not attainable with other conventional immunoaf-  
finity techniques. This immunoaffinity chromatography  
step wherein the impurities are bound to their antibodies,  
while the valuable protein is excluded in the effluent, is  
referred to as Reverse Immunoaffinity Chromatography (RIAC).

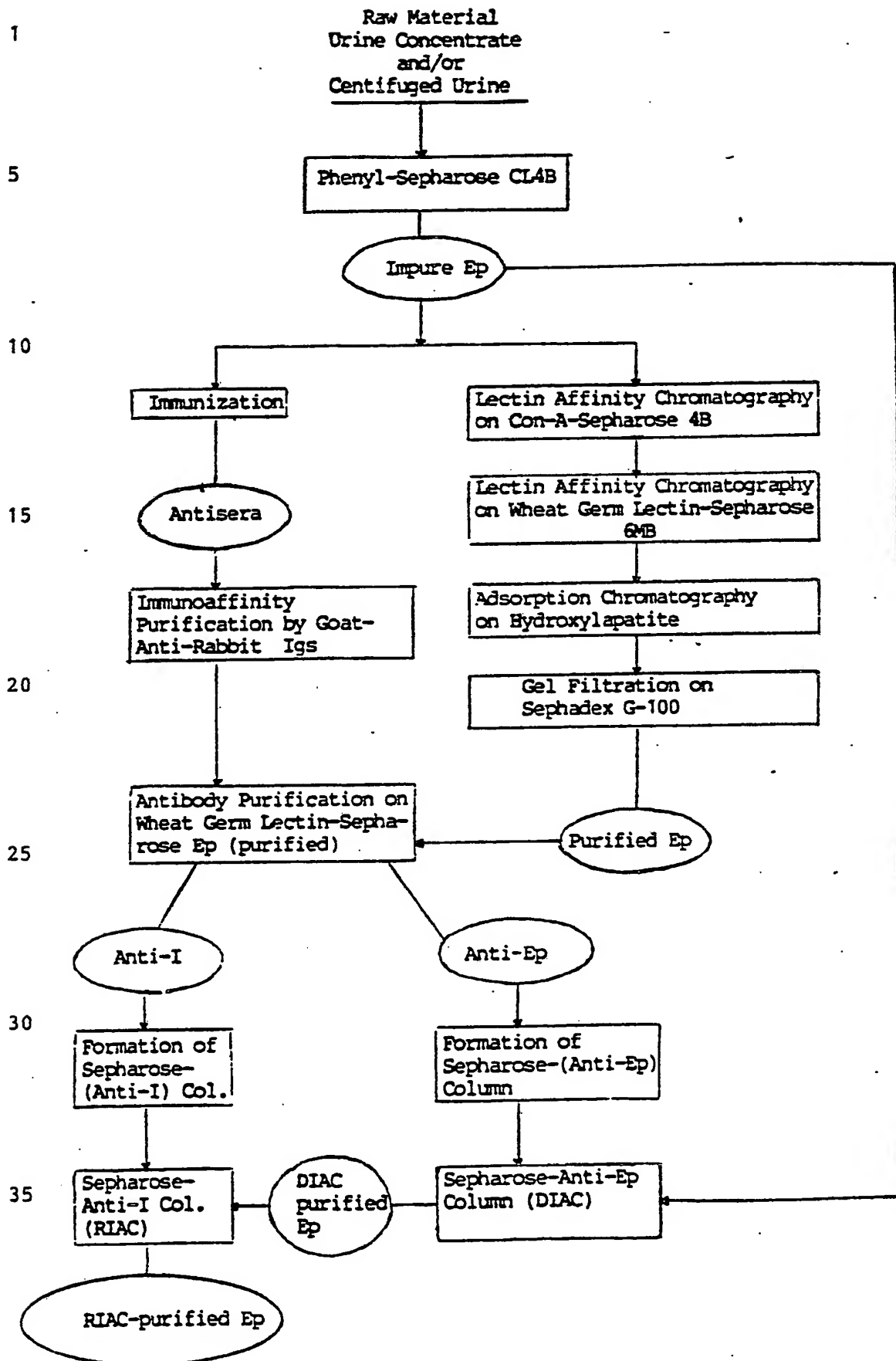
The impurities removed in the reverse immunoaf-  
10 finity step are a constant set of residual urinary contami-  
nants; and they have been copurified with Ep in many  
separation techniques, and are therefore fairly uniform  
from batch to batch. Thus, crude urine from a source  
different from that employed to generate the antisera can  
15 be effectively purified by the HIC-DIAC-RIAC procedure.  
The amount of Anti-I required for immunoadsorption of these  
minor impurities of DIAC-purified Ep is small relative to  
the total capacity of the Sepharose-(Anti I) column.  
Furthermore, since reverse immunoaffinity chromatography  
20 immunoadsorbs only the contaminating impurities, no desorp-  
tion of Ep is required, thus minimizing manipulation of  
valuable samples and increasing yield accordingly. The  
impurities retained on the column can be subsequently  
dissociated from the immunoadsorbent by eluting with an  
25 appropriate acidic eluent. The column is thus regenerated  
and ready for subsequent use.

A flow chart outlining the various steps of the  
present invention is set forth on the following page.

30

35

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1           The DIAC-RIAC purified Ep can be tested for  
homogeneity by attempting further purification using con-  
ventional purification techniques (preferably chromato-  
graphic techniques and/or gel filtration), and assayed for  
5 biological activity.

          The DIAC-RIAC purified Ep is further tested for  
homogeneity and characterized by electrophoretic techniques,  
such as gel electrophoresis, isoelectric focusing, and disc  
electrophoresis in non-dissociating systems according to  
10 well-known methods described by: (a) Laemmli, U.K.: Cleavage  
of Structural Proteins During the Assembly of the Head of  
Bacteriophage T<sub>4</sub>, Nature 227: 680-685, 1970; (b) Catsim-  
poolas, N. et al (Ed.); Biological and Biomedical Applica-  
tion of Isoelectric Focusing, New York, Plenum Press, 1977,  
15 and (c) Davis, B.J.: Disc Electrophoresis-II: Method and  
Application to Human Serum Protein, Ann. N.Y. Acad. Sci.  
121:404-427, 1964.

          The following examples serve further to illustrate  
the present invention, but not to limit its scope.

20           Materials: Phenyl-Sepharose CL4B, ConA-Sepharose  
4B, Wheat germ Lectin-Sepharose 6MB, CNBr Activated Sepha-  
rose 4B Sephadex G100 were obtained from Pharmacia Labora-  
tories, Inc., (Piscataway, New Jersey). Guanidine hydro-  
chloride (ultra-pure) was obtained from Schwartz-Mann  
25 Biochemicals (Spring Valley, New York). Ethylene glycol  
and N-acetylglucosamine were from Sigma Chemical Company  
(St. Louis, Missouri). All other chemicals were from  
Fisher Scientific Company (Fairlawn, New Jersey), except  
when otherwise specifically indicated.

30           Concentration of all column eluates was carried  
out at 4°C using an Amicon ultrafiltration apparatus with  
YM10 membrane unless otherwise specified.

35

APPENDIX BMonoclonal Anti-Ep

Ep is a weak immunogen. Accordingly, availability of pure Ep and choice of the initial immunization procedure (animals, sites and schedules) may substantially affect the efficiency of hybridoma production and the ability of the hybridoma to reliably secrete monoclonal Anti-Ep having the requisite properties.

Human Ep isolated from urine of anemic patients by Hydrophobic Interaction Chromatography on Phenyl-Sepharose CL4B and subsequently purified by Direct Immunoaffinity Chromatography followed by Reverse Immunoaffinity Chromatography (as described in the Ep Purification Patent Application) is used as the immunogen. The purity of the thus purified Ep was compared to that of homogeneous Ep provided by another investigator. The results are shown in Figure 1. The material used in this work as the antigen also shows a single band by gel isoelectric focusing and by electrophoresis under nondissociating conditions as described fully in the Ep Purification Patent Application.

In vitro immunization and subsequent fusion resulted in unstable hybrids secreting IgM. The combination of in vivo primary injection and in vitro boosting also resulted in unstable hybrids.

In vivo immunization is, therefore, preferred. Female laboratory mice are preferably used. Multiple injections in multiple sites increase the probability of obtaining a satisfactory immune response. Immune response is assayed, preferably, by Solid Phase Radioimmunoassay (SPRIA) according to the procedure of Klinman, N.R. "The Mechanism of Antigenic Stimulation of Primary and Secondary Clonal Precursor Cells" J. Exp. Med. 135:241-260 (1972), as set forth in detail in Example 2, below.

The Ep-neutralizing ability of immune mouse serum is preferably assayed by the in vivo exhypoxic polycythemic

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1 mouse method, as described in the Ep Purification Patent  
Application, as said method is the most reliable (though  
also the most expensive and time-consuming). The mice with  
an acceptable immune response are selected. Immune re-  
5 sponse is measured by Anti-Ep titer and Ep-neutralizing  
titer. For the response to be considered acceptable,  
Anti-Ep titer should show at least 50% binding at 1:10,000  
serial dilution (Figure 1A) and Ep-neutralizing titer  
should show neutralization of over 100 Ep units/ml of mouse  
10 serum. These performance characteristics are rather  
formidable, considering the weak immunogenic properties of  
Ep. Accordingly, the Ep used for immunization should be  
the purest possible and the number of mice immunized should  
be relatively large. Generally, assuming careful selection  
15 and execution of the immunization protocol, about one mouse  
in six immunized will exhibit an acceptable immune response.

Hybridomas are fused from spleen lymphocytes of  
the mice showing acceptable immune response and from non-  
secreting mouse myeloma cells. NS-1, a non-immunoglobulin  
20 secreting myeloma cell line of Balb/c origin, commercially  
available (from Mutant Cell Repository, Institute of Medical  
Research, Camden, N.J.) and resistant to 8-azaguanine is  
preferred. Other types of myeloma cell lines that, in  
principle, would also be suitable include those discussed  
25 in Kohler, G., Howe, S.C., and Milstein, K.; Fusion  
Between Immunoglobulin-Secreting and Non-Secreting Myeloma  
Cell Lines, Eur. J. Immunol. 1976, 6:292-295.

Fusion is carried out according to the general pro-  
cedure of Kennett, R.H. in Monoclonal Antibodies, Hybri-  
30 domas: A New Dimension in Biol. Analyses (Kennett et  
al, Eds.) Plenum Press, 365-367 (1980), with such modifica-  
tions as are set forth in Example 2, below.

Fusion hybrids are fed and grown in selective  
media. Surviving hybridoma cells from these media are  
35 propagated in culture and their culture media are screened  
for the presence of Anti-Ep by solid phase radioimmunoassay

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1 (SPRIA) in accordance with the method of Klinman, supra.  
Positive hybridoma cultures are then assayed for Ep-neutralization, preferably by the exhypoxic polycythemic mouse method.

5 After Ep-binding and/or Ep-neutralization have been confirmed, the positive hybridoma cultures are immediately cloned and grown in liquid media. Immediate cloning and recloning are necessary to ensure hybridoma stability. It is customary in the art not to consider a  
10 hybridoma culture stable until 100% of clone colonies derived from positive cultures upon recloning are also positive. The stability of hybridoma cells of the present invention has been confirmed by prolonged storage (1 to 2 years) without decline in Anti-Ep secretion. Three  
15 stable clones were thus isolated (hereinafter designated as 7A7, 7B9, and 2A10) from 6460 hybridomas in a total of 10 fusions.

After successful cloning of Anti-Ep-secreting hybridomas, large quantities of high-titered antibodies can  
20 be obtained by growing hybridomas in culture, or, preferably, by ascites induction. Ascites tumor can be induced by intraperitoneal injection of cloned hybridoma cells into pristane primed syngeneic mice (about  $10^7$  hybridoma cells per primed mouse). After ascites has been allowed to  
25 develop for several (preferably 2-3) weeks, ascitic fluid is harvested from the peritoneal cavity (multiple collections are possible and desirable, since the objective is to obtain as much hybridoma as possible). Monoclonal Anti-Ep activity of ascitic fluid is tested by SPRIA.

30 In order to confirm that the ascites Anti-Ep activity is in fact due to immunoglobulin, and in order to prepare stable antibody preparations, free of degradative enzymes such as proteases and nucleases, suitable for Ep-purification, for Ep mRNA identification and screening  
35 of an Ep-cDNA library, the ascites immunoglobulin must be purified. Ascites immunoglobulin and its subclass are

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1 characterized to help design and develop the most effective purification procedures.

The types of immunoglobulin chains are characterized, preferably by use of an isotype-specific rabbit  
5 Anti-mouse immunoglobulin kit (Boehringer-Mannheim, Indianapolis, Indiana). This test is also used to confirm the results after immunoglobulin purification.

The types of the three antibodies corresponding to the three stable clones produced in this work were:  
10 IgG2a/k (from 7A7), and IgG1/k (from 7B9 and 2A10).

The antibody purification technique was subject to optimization, as many of the available methods presented serious drawbacks.

Protein A-Sepharose affinity chromatography is  
15 generally satisfactory for the purification of IgG2a/k. However, this affinity adsorbent fails to bind IgG1 efficiently (even at a slightly basic pH such as 8.4). Ammonium sulfate fractionation, followed by diethylaminoethyl cellulose (DEAE-cellulose) ionic exchange and Sephadex  
20 G-200 gel filtration, fails to remove all contaminating proteases and nucleases. These degradative enzymes are deleterious to the antibodies, to Ep activity (during immunoaffinity chromatography purification using monoclonal Anti-Ep as the adsorbent) and to Ep-mRNA stability (during  
25 purification by polysome immunoprecipitation). DEAE-Affi gel blue chromatography does remove protease and RNase effectively but is difficult to optimize (maximum resolution conditions have to be determined separately for each antibody) and often results in low yields. Protein A-Sepharose  
30 CL4B affinity chromatography followed by precipitation of the eluted IgG2a with ammonium sulfate, is used for purification of monoclonal Anti-Ep 7A7. The yield is usually at least 90%. Affinity purified goat anti-mouse immunoglobulin covalently coupled to Sepharose 4B is effective for IgG1  
35 purification and remove protease and RNase. It can thus be used for the purification of monoclonal Anti-Ep 7B9 and

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1 Anti-Ep 2A10 (IgG1/k). Both monoclonal preparations can  
thus be free of detectable degradative enzyme activity.  
The thus recovered purified immunoglobulins can be quanti-  
tated by SPRIA (by measuring their native Ep-binding  
5 ability) and their heavy and light chains can be charac-  
terized by SDS-PAGE. Ep-neutralization ability can be  
tested in vivo, by exhypoxic polycythemic mouse bioassay.  
Preferably, native Ep binding is determined by immunoblot  
and  $^{125}\text{I}$ -Ep (denatured) binding is determined by immuno-  
10 precipitation.

Hybridoma 7A7 is a much stronger Anti-Ep producer  
than either 7B9 or 2A10, secreting more than ten times as  
much antibody. Of the three antibodies isolated in this  
work, only 2A10 neutralizes Ep. All three antibodies  
15 recognize (bind to) both native and  $^{125}\text{I}$ -labeled Ep.

The difference in the binding affinity between  
the monoclonal antibodies of the present invention and the  
antibody reported by Weiss, et al., supra, is illustrated  
by the fact that, in competitive radio-immunoprecipitation  
20 of  $^{125}\text{I}$ -Ep with unlabeled Ep, a ratio of native to  
labeled Ep of 41 was sufficient to cause 50% inhibition of  
 $^{125}\text{I}$ -Ep binding, (as opposed to a ratio of 2500 reported  
in Weiss et al). The increased binding affinity of the an-  
tibodies of the present invention is extremely significant  
25 because it permits the non-neutralizing antibodies disclosed  
herein to be used in Ep purification by immunoaffinity  
chromatography using purified monoclonal Anti-Ep as the  
immunoabsorbent. Immunoglobulin from 7A7(IgG2a) is pre-  
ferred because it is secreted in larger quantities, it can  
be effectively purified (with a high yield), and it does  
30 not neutralize (and thus possibly inactivate) Ep.

Purification of Ep by immunoaffinity chromatog-  
raphy (using monoclonal Anti-Ep from 7A7 as the immunoabsorbent) yields, in one step, Ep of purity and yield  
comparable to those obtained with a combination of DIAC and  
35 RIAC. Furthermore, since these monoclonal antibodies also

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1 recognize Ep from other mammals such as sheep, rabbit, rat  
and mouse, they would also be very useful in affinity  
purification of Ep from those and other species.

5 In addition to providing improved means for Ep  
purification, the production and purification of monoclonal  
Anti-Ep according to the present invention is expected to  
facilitate studies on erythropoiesis. The monoclonal  
Anti-Ep of the present invention provides useful probes for  
10 detection of Ep-synthesizing cells, Ep-responsive cells and  
Ep specific receptors, and can be used advantageously in  
conjunction with immunofluorescence and radioimmunoassay  
techniques. The regulation of Ep-gene expression under  
both normal and pathological states can be studied by the  
15 identification of Ep-mRNA and its translation product using  
immunoblotting, immunoprecipitation and immunoautoradio-  
graphy. Moreover, the monoclonal antibodies of the present  
invention are useful probes in the cloning of Ep-gene and  
in the identification of the cloned Ep-gene product. Many  
of the above uses of monoclonal Anti-Ep have been confirmed  
20 in practice, as more fully set forth in the Ep Clone Patent  
Application.

The present invention is further described in the  
following Examples which are intended to illustrate it but  
not to limit its scope.

25 Materials and Sources:

Fetal Calf Serum (FCS) and tissue culture media were  
from Gibco, Grand Island, N.Y.

Sodium azide was from Sigma, St. Louis, Mo.

Bovine serum albumin (BSA) was from Schwartz Mann,  
30 Spring Valley, N.Y.; and

<sup>59</sup>FeCl<sub>3</sub> was from New England Nuclear, Boston, Mass.

The Antigen: Human Ep was isolated from urine of anemic  
patients by Hydrophobic Interaction Chromatography on  
Phenyl-Sepharose CL4B and subsequently purified by Direct  
35 and Reverse Immunoaffinity Chromatography (DIAC-RIAC).

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What is claimed is:

1. A peptide immunochemically reactive with a monoclonal antibody to human erythropoietin.
2. A peptide according to claim 1, produced by an organism, containing at least one DNA fragment comprising a DNA sequence coding for said peptide.
3. A peptide according to claim 2, wherein said organism has been transformed by an expression vector comprising said DNA fragment.
4. A fusion protein produced by an organism, said protein consisting essentially of a peptide immunochemically reactive with a monoclonal antibody to human erythropoietin and a portion of an endogenous protein of the organism.
5. A DNA fragment having a deoxynucleotide sequence encoding a peptide immunochemically reactive with a monoclonal antibody to human erythropoietin.
6. A recombinant DNA molecule comprising a DNA fragment according to claim 5.
7. An organism transformed by a recombinant DNA molecule, according to claim 6.
8. An organism according to claim 7 comprising a bacterium of the species Escherichia coli.
9. A recombinant DNA molecule according to claim 6 containing said DNA fragment at a site within said molecule suitable for expression of the peptide coded for by said DNA fragment.

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10. A recombinant DNA molecule according to claim 9 wherein said peptide is capable of expression as a fusion protein.

11. A recombinant molecule according to claim 6 wherein said recombinant DNA molecule is a hybrid plasmid derived from pBR322.

12. A recombinant molecule according to claim 11 wherein said DNA fragment is inserted in the Providencia stuartii I cleavage site of said plasmid.

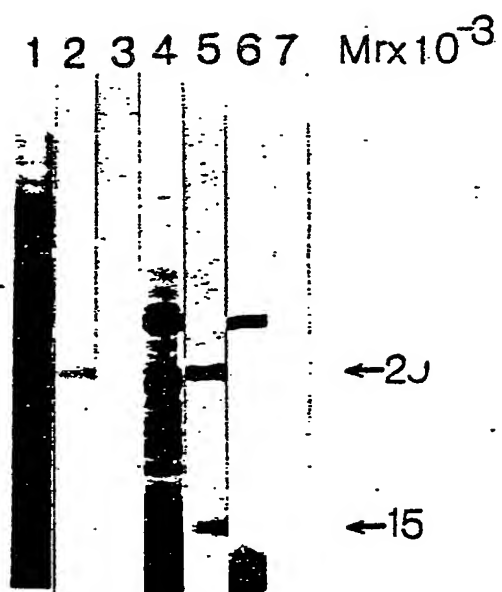
13. A recombinant DNA molecule according to claim 12, wherein said DNA fragment is inserted by homopolymeric dC:dG tailing.

14. A functional mRNA molecule carrying human erythropoietin message, in purified form.

15. A functional mRNA molecule according to claim 14, derived from a renal carcinoma cell, said renal carcinoma having a high erythropoietin titer.

16. A functional mRNA molecule according to claim 15, wherein said titer is greater than about 1 unit/g of tissue.

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**FIG. 1**

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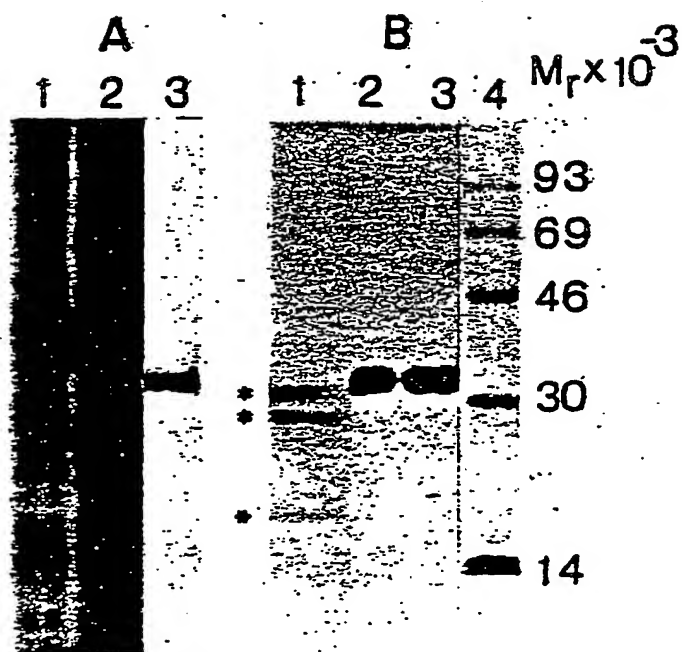
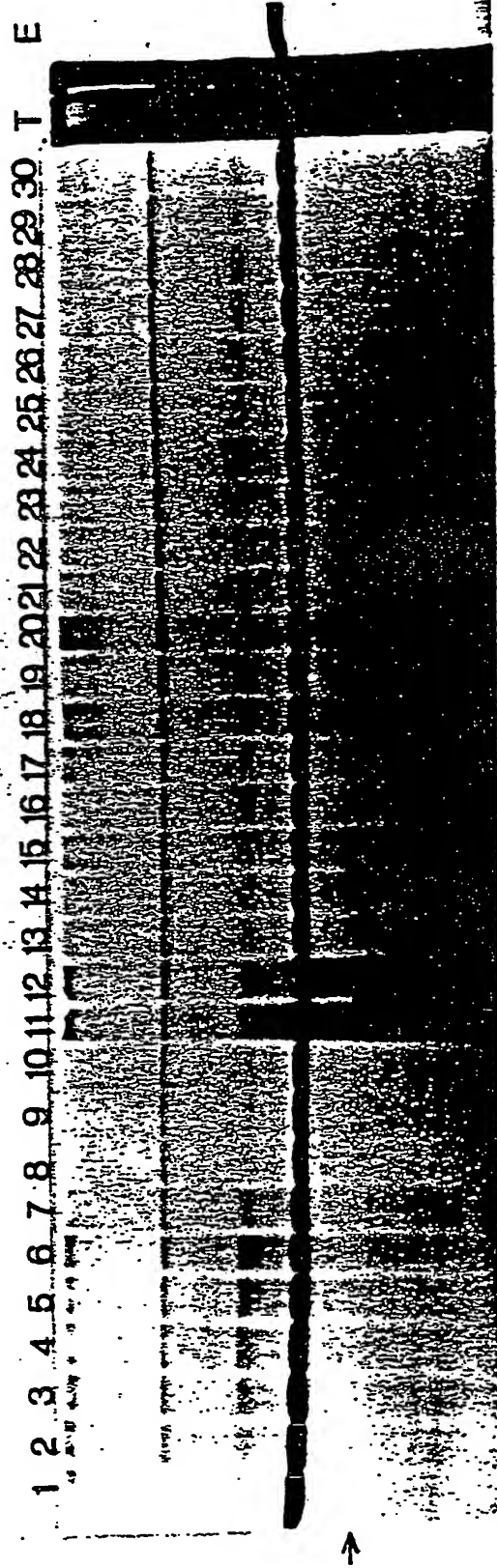
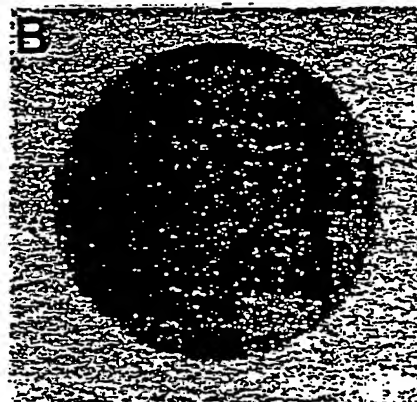
**FIG. 2**

FIG. 3

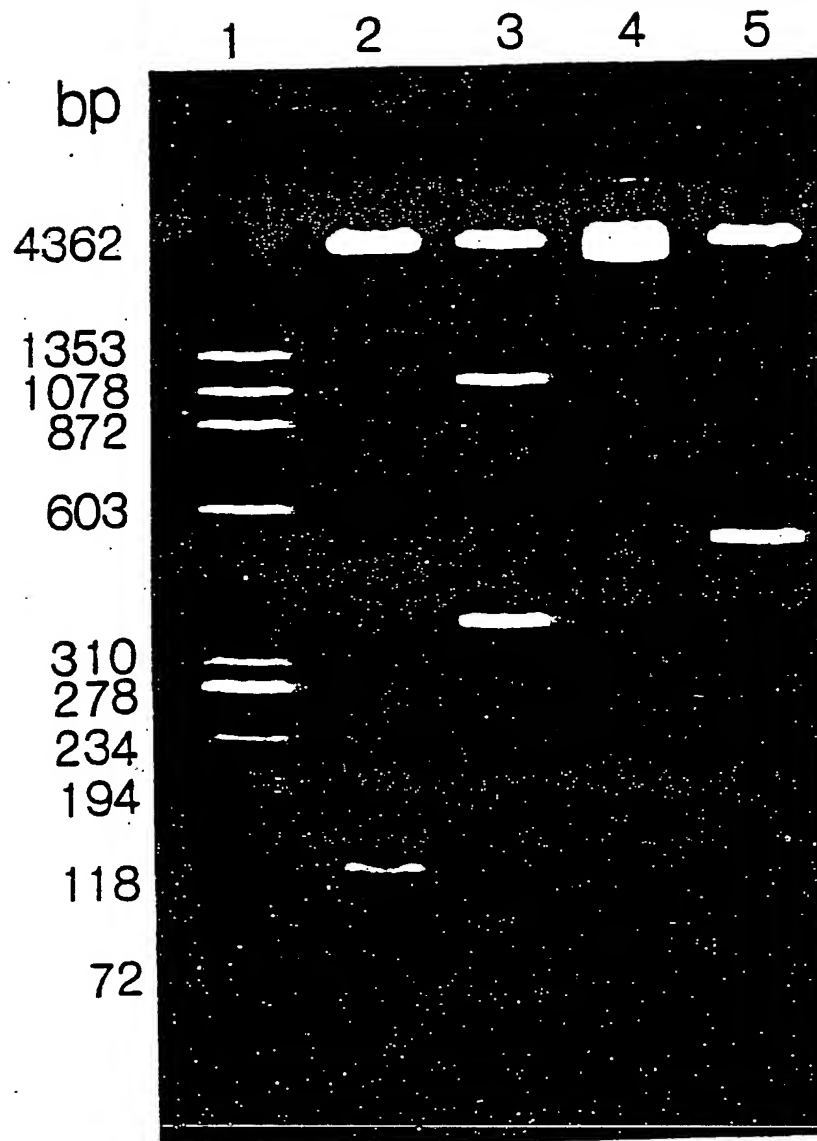


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**FIG. 4**



5 / 7

**FIG. 5**

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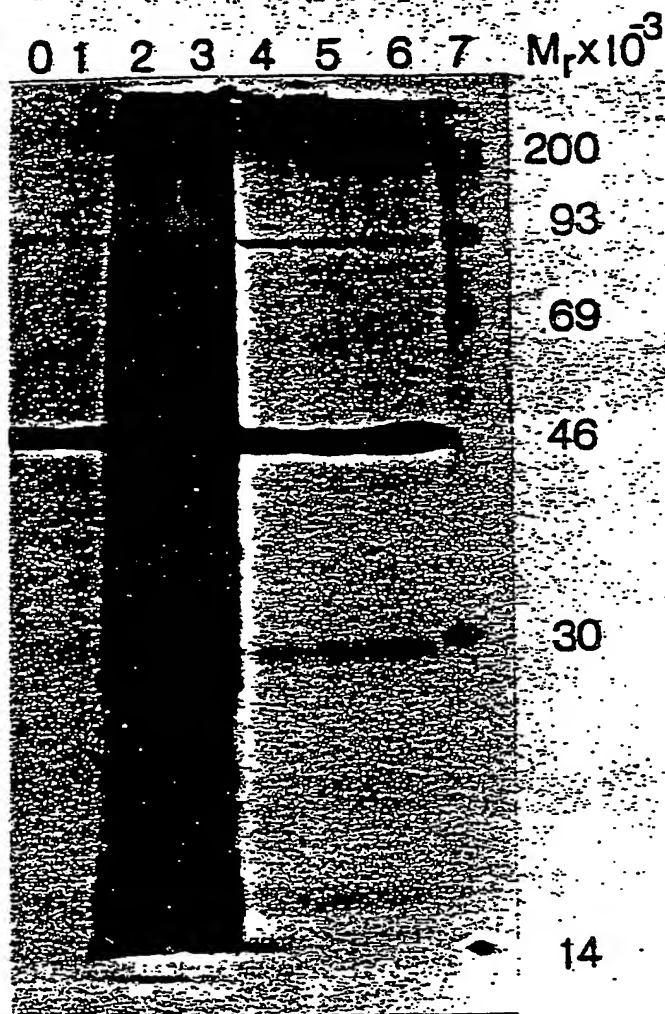
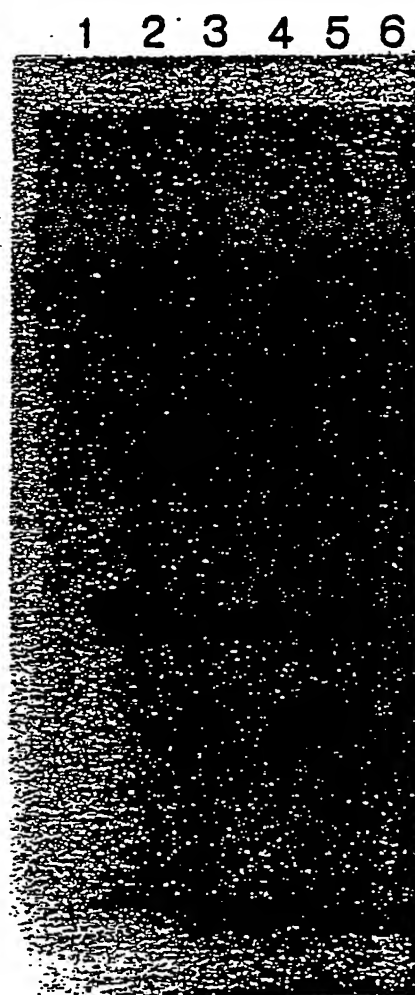
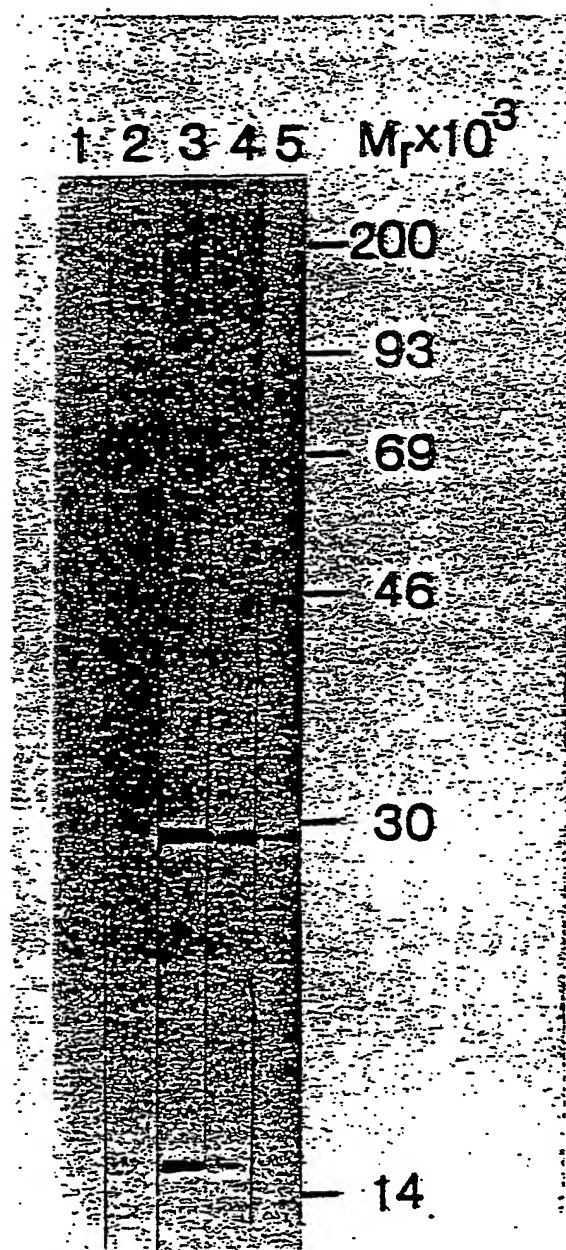
**FIG. 6A****FIG. 6B**

FIG. 7



# INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/00054

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC <sup>4</sup>		
260/112, 536/27, 435/172.3 C07E 15/12 C12N 15/00		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>6</sup>		
Classification System	Classification Symbols	
435	68, 172.3, 317	
536	27	
260	112	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>6</sup>		
Computer Search-Biosis; Chemical Abstract Index		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category <sup>5</sup>	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
T	Lee-Huang 1984 "Cloning and Expression of Human Erythropoietin cDNA in <i>E. coli</i> " Proc. Natl. Acad. Sci. V 81 p2708-12	1-16
Y	Lee-Huang 1982 (Abstract) "Monoclonal Antibodies to Human Erythropoietin" Fe. Proc. V 41 p520	1-16
X	Lin et al 1984. (Abstract) "Cloning of the Monkey Erythropoietin Gene" J. Cell. Biochem (Supp 8B) p45	5-16
Y	UK Application 2,085,887 6 May 1982 Hayashibara et al "Process for the Production of Human Erythropoietin"	1-4
Y	US 4,377,513 22 Mar 1983 Sugimoto et al. "Process for the Production of Human Erythropoietin"	1-4
<p>• Special categories of cited documents: <sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"d" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>1</sup>	Date of Mailing of this International Search Report <sup>2</sup>	
May 6, 1985	07 JUN 1985	
International Searching Authority <sup>1</sup>	Signature of Authorized Officer <sup>10</sup>	
ISA/US	Jeanne M. Gieser	

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y US 4,503,151 Paddock et al 5 Mar. 1985  
 "Recombinant cDNA Construction Method and  
 Hybrid Nucleotides useful in Cloning"

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V ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>10</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:

2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>13</sup>, specifically:

VI ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>11</sup>

This International Searching Authority found multiple inventions in this international application as follows:

Claims 1-4 drawn to a peptide

Claims 5-6 drawn to DNA, RNA, and microorganism

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

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